Molecular Biology of the Cell
Vol. 17, 213–226, January 2006

A Genomewide Screen for Petite-negative Yeast Strains Yields a New Subunit of the i-AAA Protease Complex[D]

Cory D. Dunn,* Marina S. Lee,†‡ Forrest A. Spencer,† and Robert E. Jensen*

*Department of Cell Biology and †McKusick-Nathans Institute of Genetic Medicine, The Johns Hopkins University School of Medicine, Baltimore, MD 21205

Submitted June 30, 2005; Revised October 19, 2005; Accepted October 21, 2005
Monitoring Editor: Thomas Fox

Unlike many other organisms, the yeast *Saccharomyces cerevisiae* can tolerate the loss of mitochondrial DNA (mtDNA). Although a few proteins have been identified that are required for yeast cell viability without mtDNA, the mechanism of mtDNA-independent growth is not completely understood. To probe the relationship between the mitochondrial genome and cell viability, we conducted a microarray-based, genomewide screen for mitochondrial DNA-dependent yeast mutants. Among the several genes that we discovered is *MGR1*, which encodes a novel subunit of the i-AAA protease complex located in the mitochondrial inner membrane. *mgr1*Δ mutants retain some i-AAA protease activity, yet mitochondria lacking Mgr1p contain a misassembled i-AAA protease and are defective for turnover of mitochondrial inner membrane proteins. Our results highlight the importance of the i-AAA complex and proteolysis at the inner membrane in cells lacking mitochondrial DNA.

INTRODUCTION

Mitochondria are found in virtually all eukaryotic cells, playing key roles in diverse processes such as ATP synthesis, ion homeostasis, lipid metabolism, cell fate determination, apoptosis, and aging (Tzagoloff *et al.*, 1994; Green and Kroemer, 2004; Trifunovic *et al.*, 2004). The ~1000 proteins that make up a mitochondrion (Mootha *et al.*, 2003; Sickmann *et al.*, 2003; Jensen *et al.*, 2004) are encoded either in the nucleus or by mitochondrial DNA (mtDNA). The vast majority of these proteins are encoded in the nuclear genome, synthesized in the cytosol, and then imported into one of four mitochondrial locations: the outer membrane (OM), the intermembrane space (IMS), the inner membrane (IM) or the matrix (Rehling *et al.*, 2004). mtDNA encodes only a small number of proteins, most of which are subunits of the large, inner membrane complexes that mediate oxidative phosphorylation (Attardi and Schatz, 1988).

The yeast *S. cerevisiae* can grow in the absence of mtDNA. Yeast strains that contain wild-type mtDNA, or "rho$^+$" cells, are able to respire and can grow on nonfermentable media (Tzagoloff, 1982). Cells that contain nonfunctional, mutated mtDNA (rho$^-$) or have completely lost their mtDNA (rho^0) are called "cytoplasmic petite" mutants. Because rho^0 cells lack a mitochondrial genome, they lack functional mtDNA-encoded subunits of both the electron transport chain and the ATP synthase, and they grow only by glycolysis on fermentable carbon sources such as glucose (Tzagoloff, 1982). The inner membrane electrochemical potential ($\Delta\Psi$) is required for mitochondrial ATP synthesis, for the transport of many small molecules into and out of the matrix (Tzagoloff, 1982), and for the import of proteins from the cytosol (Rehling *et al.*, 2004). In yeast cells with intact mtDNA, the IM potential is largely produced by the electron transport chain, which pumps protons across the IM to the IMS (Tzagoloff, 1982). However, in cells without mtDNA, $\Delta\Psi$ appears to be maintained by electrogenic exchange of ATP^{-4} generated by glycolysis for ADP^{-3} produced by the F_1-ATPase (Dupont *et al.*, 1985; Giraud and Velours, 1997). This model comes from the observations that yeast mutants lacking the major ATP/ADP carrier (Kovacova *et al.*, 1968) or cells without functional F_1-ATPase (Ebner and Schatz, 1973; Chen and Clark-Walker, 1999) cannot live without their mtDNA, even when they are grown on glucose-containing medium. These mtDNA-dependent yeast cells are called "petite-negative" mutants (Chen and Clark-Walker, 2000).

Defects in several other proteins cause a petite-negative phenotype. For example, we reported that yeast lacking the mitochondrial protein import components Tim18p and Tom70p do not grow without functional mtDNA (Kerscher *et al.*, 2000; Dunn and Jensen, 2003). Others have demonstrated that yeast mutated for import components Tim9p, Tim10p, and Tim12p are unable to live without mtDNA (Senapin *et al.*, 2003). However, the relationship between these different import components and mtDNA requirement remains unclear. Yeast mutants deficient in Yme1p, an ATP- and zinc-dependent protease located in the IM (Nakai *et al.*, 1995; Leonhard *et al.*, 1996; Weber *et al.*, 1996), also exhibit a severe growth defect on glucose medium in the absence of mtDNA (Thorsness *et al.*, 1993). Yme1p is found in a large structure in the IM, called the "i-AAA protease complex," which may play an important role in the F_1-ATPase activity of rho^0 cells (Weber *et al.*, 1995; Kominsky *et al.*, 2002). To further understand the requirement for the mitochondrial genome in yeast, we conducted a genomewide, microarray-based screen for petite-negative mutants. Although some of

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05–06–0585) on November 2, 2005.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

‡ Present address: Genetics and Biology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892.

Address correspondence to: Robert E. Jensen (rjensen@jhmi.edu).

the mutants that we identified have previously been described as petite-negative, several other mutants are components of cellular pathways that were not known to be required for the viability of yeast lacking mtDNA. One mutant from our screen is defective in an uncharacterized protein, which we have named Mgr1p. Mgr1p is located in the mitochondrial IM, interacts with Yme1p, and is a new subunit of the i-AAA protease complex.

MATERIALS AND METHODS

Strains, Media, and Genetic Methods

A list of the strains used in this study and their genotypes are located in Supplementary Table 1. Diploid strain RJ605 was generated by crossing FY833 (Winston *et al.*, 1995) to FY844 (Winston *et al.*, 1995). Strain RJ1970, which expresses an Mgr1p-myc fusion protein, was constructed by homologous recombination in diploid strain RJ605 using the *MYC::TRP1* cassette from pFA6a-13Myc-TRP1 (Longtine *et al.*, 1998) amplified by oligonucleotides 1620 and 1621 (see Supplementary Table 2). After sporulation, haploid strains RJ1970 (*MGR1-MYC*) and RJ1971 (*MGR1*) were isolated. Strain RJ1688 was made by transforming *mgr1*Δ strain RJ2058 with plasmid pM486 expressing *MGR1-GFP*. *mgr1*Δ strain RJ1961 was generated by gene replacement using the *TRP1* gene from plasmid pRS304 (Sikorski and Hieter, 1989), the diploid strain RJ605, and oligonucleotides 1652 and 1653. After sporulation, *mgr1*Δ strain RJ1961 and *MGR1* strain RJ1962 were isolated. *MGR1-MYC yme1*Δ strain RJ1972 and *yme1*Δ strain RJ1974 were constructed using the *URA3* gene from plasmid pRS306 (Sikorski and Hieter, 1989), primers 834 and 835, and *MGR1-MYC* strain RJ1970 or *MGR1* strain RJ1971. *MGR1-MYC YME1-HA* strain RJ1980 was constructed by homologous recombination in *MGR1-MYC* strain RJ1970 using the *HA::kanMX6* cassette from pFA6a-3HA-kanMX6 (Longtine *et al.*, 1998) amplified by oligonucleotides 1662 and 1663. *mgr1*Δ strains RJ2001 and RJ2059 were constructed using the *kanMX4* marker from pRS400 (Brachmann *et al.*, 1998), primers 1652 and 1653, and strain PTY44 (Thorsness and Fox, 1993) or strain BY4742 (Brachmann *et al.*, 1998). Similarly, *yme1*Δ strains RJ2040 and RJ2051 were made using oligonucleotides 834 and 835, and strains PTY44 or BY4742. *NDE1-HA* strain RJ2077, *mgr1*Δ *NDE1-HA* strain RJ2078, and *yme1*Δ *NDE1-HA* strain RJ2079 were generated with the *HA::kanMX6* cassette from pFA6a-3HA-kanMX6 (Longtine *et al.*, 1998), oligonucleotides 1823 and 1824, and either *mgr1*Δ strain RJ1961, *yme1*Δ strain RJ1974, or wild-type strain RJ1962. *mrpl16*Δ strains RJ1924 and RJ1925 were made using diploid strain RJ1082 (Kerscher *et al.*, 2000), the *LEU2* gene from pRS305 (Sikorski and Hieter, 1989), and primers 1559 and 1560. After sporulation, haploid *mrpl16*Δ strains RJ1924 and RJ1925 were isolated. *yta10*Δ/*YTA10 yta12*Δ/*YTA12 yme1*Δ/*YME1* strain RJ2056 was constructed by disrupting *YTA10*, using primers 836 and 837 and the *HIS3* gene from plasmid pRS303 (Sikorski and Hieter, 1989), *YME1*, using primers 834 and 835 and the *kanMX4* gene from pRS400 and *YTA12*, using primers 1784 and 1785 and the *TRP1* gene from pRS304 (Sikorski and Hieter, 1989), in diploid strain RJ605. Media and genetic techniques were as described (Adams *et al.*, 1997).

Microarray Screen for Petite-negative Deletion Mutants

A pool of cells representing ~3800 haploid yeast knockout mutants (Ooi *et al.*, 2001) was diluted to an OD_{600} of $\sim 2 \times 10^{-3}$ into 100 ml of YEPD media, or 100 ml of YEPD containing 25 μg/ml ethidium bromide (EtBr; Sigma, St. Louis, MO). Cultures of EtBr-treated and untreated cells were grown for ~18 generations at 30°C, and then 10 OD_{600} units of cells from each culture were harvested by centrifugation. After genomic DNA isolation (Adams *et al.*, 1997), probes corresponding to the DOWNTAG, a distinct 20-bp DNA sequence that uniquely identifies each YKO mutant (Shoemaker *et al.*, 1996; Winzeler *et al.*, 1999; Giaever *et al.*, 2002), were prepared by PCR amplification using oligonucleotides D1 and D2 labeled with either the Cy3 or Cy5 fluorophore. In three experiments, Cy5-labeled primers were used to amplify DOWNTAGs from 400 ng genomic DNA isolated from EtBr-treated cells, whereas Cy3-labeled primers were used to PCR amplify the DOWNTAGs of cells from the control culture. In a fourth experiment, the dyes were swapped. The PCR products were used as hybridization probes to oligonucleotide microarrays (a gift of D. Shoemaker and J. Boeke, Johns Hopkins University) containing the complementary DOWNTAG sequences from each YKO (in triplicate) as described (Shoemaker *et al.*, 1996; Winzeler *et al.*, 1999; Ooi *et al.*, 2001; Giaever *et al.*, 2002).

After hybridizations, microarray slides were scanned and analyzed as described (Lee and Spencer, 2004). Briefly, for each YKO, the mode pixel value for each spot was log2 transformed and quantile normalized (Bolstad *et al.*, 2003). For each of four independent hybridization experiments (two hybridizations for each of two experimental replicates), a summary score for each YKO was calculated by taking the median value of the triplicate DOWNTAG spots present on each slide. From these summary scores from both the Cy5 and Cy3 channels, we determined a relative ratio of hybridization between the control probe (−EtBr) and experimental probe (+EtBr). Finally, the ratios from our four hybridizations were averaged to give the final ratio for each YKO (see Supplementary Table 3). YKOs with large ratios (high relative abundance in the control sample and low relative abundance in the sample treated with EtBr) were candidates for further testing. We note that for each hybridization, YKOs with triplicate signal variance that was greater among the control signals than between the control and experimental signals were not assigned ratio scores and dropped from the analysis.

Plasmid Constructions

pM486, a *CEN6-LEU2* plasmid that expresses an Mgr1p-GFP fusion protein was constructed by PCR amplifying the *MGR1* open reading frame (ORF) and 498 base pairs of upstream sequence yeast genomic DNA (Hoffman and Winston, 1987) using oligonucleotides 1274 and 1275. The PCR fragment was digested with *Xho*I and *Not*I and inserted into *Xho*I/*Not*I-digested pAA1, which encodes the green fluorescent protein (GFP; Sesaki and Jensen, 1999). The full-length Mgr1p-GFP fusion protein was functional, rescuing the petite-negative phenotype of *mgr1*Δ cells. pT175, which expresses Mgr1p from the SP6 promoter was generated by PCR amplification of the *MGR1* ORF from genomic DNA using primers 1770 and 1771. After digestion with *Eco*RI and *Hin*dIII, the *MGR1*-containing PCR fragment was inserted into *Eco*RI/*Hin*dIII-cut pSP64 (Promega, Madison, WI). pRS315-*ATP3* (pM487) and pRS315-*ATP3-5* (pM488) were constructed by PCR amplifying the *ATP3* gene from wild-type strain PTY44 (Thorsness and Fox, 1993) and *ATP3-5* strain PTY100 (Weber *et al.*, 1995) using primers 1780 and 1781, digesting the PCR products with *Xho*I and *Not*I, and then inserting the DNA fragment into *Xho*I/*Not*I-digested pRS315 (Sikorski and Hieter, 1989).

Mitochondrial Fractionation

Mitochondria were isolated from yeast cells as described (Daum *et al.*, 1982), except that SEH buffer (250 mM sucrose, 1 mM EDTA, 20 mM HEPES-KOH, pH 7.4) with 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) and a 1:1000 dilution of a protease inhibitor cocktail (P8340; Sigma) was used. Osmotic shock of mitochondria and protease treatment of mitochondria and mitoplasts were as described (Kerscher *et al.*, 2000), except that 200 μg of mitochondria or mitoplasts at 0.2 mg/ml were used. The separation of outer membrane and inner membrane vesicles by sucrose step-gradients were as described (Pon *et al.*, 1989). Treatment of mitochondria with alkali was as described (Kerscher *et al.*, 2000), except that treated samples were centrifuged for 60 min at 100,000 × *g*.

Proteins were analyzed by SDS-PAGE. Western blots to Immobilon filters (Millipore, Billerica, MA) were performed using standard techniques (Current Protocols Online; http://www.mrw2.interscience.wiley.com/cponline). HA-tagged proteins were identified by incubation of filters with mouse ascites fluid prepared using 12CA5 cells (Niman *et al.*, 1983); BABCO, Berkeley, CA), and myc-tagged proteins were visualized using mouse ascites fluid prepared using 9E10 cells (Evan *et al.*, 1985); Covance, Denver, PA). Mitochondrial proteins were identified using antiserum to Tim23p (Emtage and Jensen, 1993), Mas2p (Jensen and Yaffe, 1988), Yme1p (Thorsness *et al.*, 1993), Phb1p (Steglich *et al.*, 1999), and Phb2p (Steglich *et al.*, 1999). Immune complexes were visualized using HRP-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) followed by chemiluminescence (West Pico; Pierce, Biotechnology, Rockford, IL). Western blots were imaged using a Versadoc Imaging System and Quantity One software (Bio-Rad, Hercules, CA).

Import into Isolated Mitochondria

[^{35}S]methionine-labeled mitochondrial precursor proteins were synthesized using the TNT SP6-Quick Coupled Transcription/Translation System (Promega, Fitchburg, WI) and added to 3–5% of total import reaction. pT58, which was used to produce su9-DHFR, consists of a fusion between amino acids 1–69 of *Neurospora crassa* mitochondrial ATPase subunit 9 and mouse DHFR (Pfanner *et al.*, 1987), and pT176, which encodes a fusion of amino acids 1–161 of Yta10p to $DHFR^{MUT}$ (Leonhard *et al.*, 1999) have been described. Imports were performed in import buffer (0.6 M sorbitol, 50 mM HEPES-KOH, pH 7.4, 25 mM KCl, 10 mM $MgCl_2$, 2 mM KPO_4, pH 7.4, 0.5 mM EDTA, 1 mg/ml BSA, 2 mM ATP, 2 mM NADH) containing an ATP regeneration system (100 μg/ml creatine kinase [Sigma], 5 mM creatine phosphate [Sigma]). Import reactions were stopped by incubation on ice and/or by the addition of proteinase K. For imports into mitochondria lacking inner membrane potential, NADH was replaced by 1 μM valinomycin (Sigma). After centrifugation, import reactions were analyzed by SDS-PAGE, and ^{35}S-labeled proteins were detected and quantitated by phosphorimaging using a Molecular Imager FX (Bio-Rad) and Quantity One software.

Analysis of the Mgr1p-containing Complex

Agarose beads coupled to HA or myc antibodies (Niman *et al.*, 1983; Evan *et al.*, 1985) were prepared using the Seize Immunoprecipitation Kit (Pierce Chemical, Rockford, IL) according to manufacturer's instructions. Immunoprecipitations from detergent-solubilized mitochondria were as described (Sesaki *et al.*, 2003), except that 0.5 mg of mitochondria was solubilized for 10 min in 1 ml of 1% digitonin, 50 mM NaCl, 30 mM HEPES-KOH, pH 7.4, containing a 1:1000 dilution of protease inhibitor cocktail and 1 mM PMSF.

After removal of insoluble material by centrifugation at 12,500 × *g* for 10 min, mitochondrial lysates were incubated with antibody-coupled beads at 4°C for 4 h with gentle agitation. Sample beads were washed three times with 0.1% digitonin, 50 mM NaCl, 30 mM HEPES-KOH, pH 7.4, containing protease inhibitors before beads were resuspended and boiled for 5 min in 2× SDS sample buffer (2% SDS, 20% glycerol, 10 μg/ml bromophenol blue, 200 mM Tris-HCl, pH 6.8) containing 0.6 M β-mercaptoethanol.

For blue-native electrophoresis, 600 μg mitochondria was resuspended on ice in 96 μl of 50 mM NaCl, 5 mM 6-aminocaproic acid, 100 mM Bis Tris, pH 7.0, 1× α2 macroglobulin, with a 1:1000 dilution of protease inhibitor cocktail and 1 mM PMSF. Then, 24 μl of 10% digitonin was added, and mitochondria were solubilized for 15 min. After removal of insoluble material by centrifugation at 12,500 × *g* for 10 min, 100–200 μg of mitochondrial protein was loaded and run on polyacrylamide gradient gels and Western blotted as described (Schagger and von Jagow, 1991; Arnold *et al.,* 1998; Kerscher *et al.,* 2000).

Antiserum to Mitochondrial Proteins

Antiserum to OM45p and Tom70p were raised using bacterially expressed MBP fusion proteins. For MBP-OM45p, the *OM45* ORF lacking the first 22 amino acids was PCR amplified with primers 774 and 775, digested with *Hin*dIII and *Pst*I, and inserted into *Hin*dIII/*Pst*I-cut pMAL-cR1 (New England Biolabs, Beverly, MA) to form pB63. For MBP-Tom70p, the *TOM70* ORF lacking the first 37 amino acids was PCR amplified with primers 776 and 777, digested with *Pst*I and *Hin*dIII, and inserted into *Hin*dIII/*Pst*I-cut pMAL-cR1 to form pB64. MBP fusion proteins were expressed in bacteria according to manufacturer's instructions and purified by SDS-PAGE, and antiserum to the proteins was raised in rabbits (Covance).

Microscopy

Yeast cells were grown to midlog phase in S medium containing 2% galactose and then examined with a Zeiss Axioskop microscope (Thornwood, NY) with a 100× Plan-Neofluor objective. DIC and fluorescent images were captured with an Orca ER CCD camera (Hamamatsu, Bridgewater, NJ) using Open Lab software, version 3.1.4 (Improvision, Lexington, MA).

RESULTS

A Genomewide Screen for Petite-negative Yeast Mutants

To identify new genes required for yeast cell viability in the absence of mtDNA, we performed a microarray-based screen for mutants unable to live on medium containing EtBr. Wild-type cells grown on EtBr-containing medium rapidly lose their mtDNA but continue to grow by fermentation on glucose-containing YEPD medium (Slonimski *et al.,* 1968; Goldring *et al.,* 1970). In contrast, petite-negative mutants lose viability on YEPD medium containing EtBr (Chen and Clark-Walker, 2000). Our studies with known mtDNA-requiring mutants, such as *tom70*Δ (Dunn and Jensen, 2003) showed that these cells stopped dividing in YEPD + EtBr medium after ~12–14 generations (C. Dunn, unpublished observations).

For our screen, we used a collection of haploid yeast knockouts (YKOs; Giaever *et al.,* 2002) each disrupted in a different nonessential ORF by a kanMX4 cassette conferring G418 resistance (Wach *et al.,* 1994). Because each YKO contains unique identifier sequences (20-mer "tags"), individual strains in a mutant pool can be detected by hybridization of PCR-amplified tags to oligonucleotide microarrays (Shoemaker *et al.,* 1996; Winzeler *et al.,* 1999; Giaever *et al.,* 2002). This approach efficiently allows comparison of YKO representation after growth under experimental and control conditions. A pool of haploid YKOs in YEPD medium was split into two aliquots and grown with or without 25 μg/ml EtBr for 18 generations. Genomic DNA was isolated from the treated and untreated cells, and tags were amplified using either Cy3- or Cy5- labeled primers to distinguish control and experimental pools. The PCR products were cohybridized on glass slide arrays containing tag oligonucleotide features, and the control:experimental signal ratio was determined for each YKO. For a YKO with decreased representation after EtBr culture (due to lethality or slow growth), we expected that the control:experimental signal ratio would be higher in comparison to the majority of strains in the pool, which are much less affected by loss of mtDNA. Using this strategy, we identified candidate EtBr-sensitive mutants (Figure 1A). We have used these data to construct a table of YKOs, showing those most severely affected by EtBr at the top of the table (see Supplementary Table 3)

Interestingly, we also noted a subset of YKOs that appeared less affected by EtBr treatment than the bulk of mutant strains in our pool (Figure 1A). Two examples are *fzo1*Δ, which is defective in mitochondrial fusion and rapidly loses mtDNA (Hermann *et al.,* 1998; Rapaport *et al.,* 1998), and *mrpl38*Δ, a mutant lacking a component of the mitochondrial ribosome (Grohmann *et al.,* 1991). Because these strains are already petite, their growth is not expected to be affected by EtBr. Intriguingly, we also found the *rpn4*Δ mutant, lacking a transcription factor driving proteasome synthesis (Mannhaupt *et al.,* 1999; Xie and Varshavsky, 2001), in the subset of YKOs less affected by EtBr. Unlike *fzo1*Δ and similar mutants lacking the ability to respire, *rpn4*Δ cells are not petite. We found that mutants lacking *RPN4* grow better than wild-type cells after loss of mtDNA (C. Dunn, unpublished observations).

To more fully characterize mtDNA-dependent YKOs, we took the 104 YKO strains that our microarray studies indicated were the most defective in their growth on EtBr-containing medium, and directly examined them for their ability to grow without mtDNA. We patched these 104 strains onto YEPD medium containing 25 μg/ml EtBr for 3 d at 30°C and then passaged them onto YEPD plates lacking EtBr at 30°C for another 3 d. Thirty-four mutants showed a strong growth defect after growth on EtBr (see Supplementary Table 3). The other YKOs showed a less severe or more variable growth defect after EtBr treatment and were not further analyzed. As an independent test for mtDNA-dependence, we crossed each of the 34 remaining YKOs to the *mrpl16*Δ mutant, which lacks a subunit of the mitochondrial ribosome (Pan and Mason, 1995) and is respiratory deficient. We reasoned that strains lacking the L16 subunit of mitochondrial ribosomes would be unable to translate mtDNA-encoded proteins and would therefore phenocopy a strain that has been forced to lose mtDNA by EtBr treatment. The 34 YKOs were crossed to *mrpl16*Δ, sporulated and the viability of the spores containing both the YKO disruption and the *mrpl16*Δ mutation were examined. For 12 YKOs, the double mutants showed a strong synthetic lethal growth defect, and only microcolonies formed after spore germination. These strains are denoted in Supplementary Table 3 and include three mutants, *tim18*Δ, *tom70*Δ, and *fmc1*Δ, which were previously described as petite-negative (Kerscher *et al.,* 2000; Lefebvre-Legendre *et al.,* 2001; Dunn and Jensen, 2003). Moreover, *yjr120w*Δ was found in our screen (Figure 1B), and this mutation affects expression of *ATP2*, a gene known to be required by rho^0 yeast (C. Dunn, unpublished results). We also found that cells lacking Phb1p, a component of the IM prohibitin complex, are petite-negative (Figure 1B). Cells lacking a functional prohibitin complex have previously been described as containing misshapen mitochondria after mtDNA loss (Berger and Yaffe, 1998), but *phb1*Δ has not been described as a petite-negative mutant. The petite-negative phenotype of *phb1*Δ is likely dependent upon strain background, because strains deleted of *PHB1* in a different background [FY background; (Winston *et al.,* 1995)] than that used in our screen remain viable after mtDNA loss (C. Dunn, unpublished observations). Seven other mutants were also not previously known to be mtDNA dependent (Figure 1B), including *opi1*Δ, *thr1*Δ, *thr4*Δ, *ira2*Δ, *pde2*Δ, and uncharacterized mutants *ycl044c*Δ

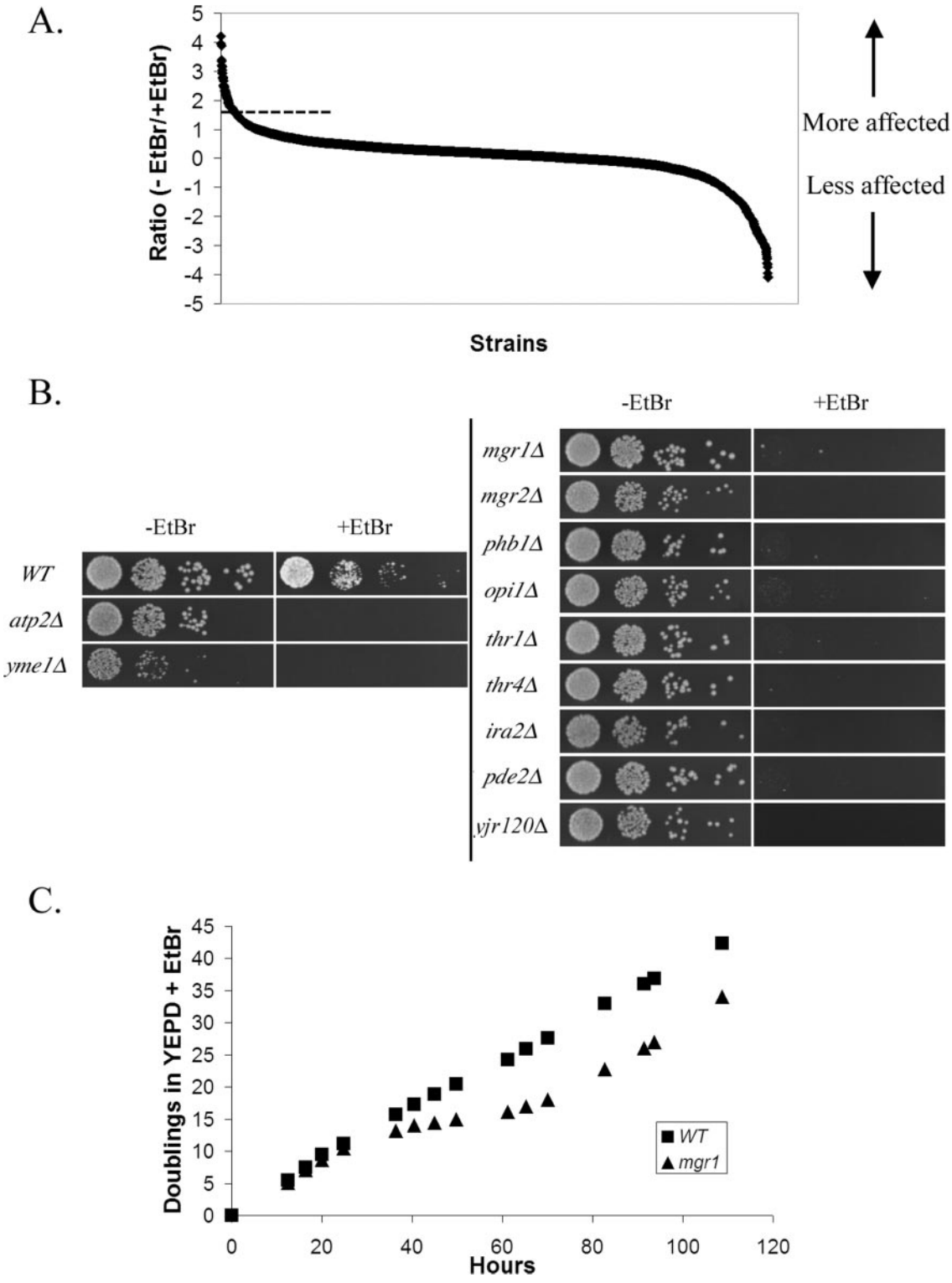

Figure 1. Yeast knockouts that require mtDNA for viability. (A) Distribution of YKOs after growth in EtBr-containing medium. In microarray analysis, the $\log_2$-converted ratio of the amount of hybridization to the probe from control cultures (−EtBr) and the probe from experimental cultures (+EtBr) for each YKO in our pool was determined. This ratio is plotted on the *Y*-axis. Thus, YKOs whose growth is most affected by EtBr are to the left, and those least affected are to the right. Strains above the dotted line were candidates for further testing. (B) Representative mutants from the genomewide screen. *mgr1*Δ (RJ2058), *phb1*Δ (RJ2083), *opi1*Δ (RJ2084), *mgr2*Δ (RJ2085), *thr1*Δ (RJ2086), *thr4*Δ (RJ2087), *ira2*Δ (RJ2088), *pde2*Δ (RJ2089), and *yjr120w*Δ (RJ2091) cells were grown 7 h in YEPD broth at 30°C without ethidium bromide (−EtBr) or for 22 h in YEPD containing 25 μg/ml EtBr (+EtBr). A 4-μl aliquot of cells at OD_{600} of 0.02, and serial 10-fold dilutions thereof, were spotted onto YEPD (−EtBr) or YEPD + EtBr (+EtBr) plates and grown at 30°C for 2 d. As controls, wild-type strain BY4742 and two known petite-negative mutants, *atp2*Δ (RJ1577) and *yme1*Δ (RJ2051) were also examined. (C) Growth of *mgr1*Δ cells is defective in EtBr medium. *WT* (BY4742) and *mgr1*Δ (RJ2058) strains were inoculated into YEPD medium and then transferred to YEPD with 25 μg/ml EtBr. At indicated times, the OD_{600} of each culture was determined and the number of doublings was calculated.

and *ypl098c*Δ. We refer to *YCL044C* as *MGR1* (Mitochondrial Genome Required) and to *YPL098C* as *MGR2*. We focus our efforts below on *MGR1*.

MGR1 Is Required for the Normal Growth of Yeast Cells Lacking mtDNA

Like the known petite-negative mutants, *atp2*Δ (Chen and Clark-Walker, 1999) and *yme1*Δ (Thorsness *et al.*, 1993), *mgr1*Δ cells cannot lose their mtDNA and fail to grow after treatment with EtBr (Figure 1B). When wild-type and *mgr1*Δ strains were transferred from YEPD medium lacking EtBr into medium containing 25 μg/ml EtBr, we found that the doubling times of wild-type and *mgr1*Δ strains were equivalent for ~25–30 h (Figure 1C). After this time, wild-type cells continued to divide at a constant rate, whereas the *mgr1*Δ cells stopped dividing.

We noticed that at much later times (~70 h) the *mgr1*Δ culture began to recover and attained a wild-type growth profile between 80 and 110 h. We postulated that this recovery might be due to the accumulation of bypass mutations. We isolated six viable *mgr1*Δ rho^0 cells after culture in YEPD + EtBr and crossed them to wild-type cells. After meiotic analysis, we found that each of the six isolates contained a defect in a single nuclear-encoded gene. Two isolates carried recessive suppressors, and four isolates contained dominant suppressors (C. Dunn, unpublished results). We postulate that EtBr is stimulating the rapid accumulation of nuclear mutations in our *mgr1*Δ cultures. Supporting this view, we found a number of YKOs defective in nuclear DNA repair, such as *RAD57*, *MUS81*, *MMS4*, *XRS2*, *RAD54*, *RAD59*, *MMS1*, *RAD55*, and *RAD53* (Supplementary Table 3), in our microarray screen. Because these repair mutants are not petite-negative, it is likely that EtBr mutagenizes nuclear DNA even at the concentrations used to remove functional mtDNA and thereby promotes the accumulation of *mgr1*Δ suppressors. Interestingly, we also found that the *mgr1*Δ petite-negative phenotype is variable according to genetic background; *mgr1*Δ was not required for viability of ρ^0 cells derived from strain PTY44 (Supplementary Table 1; C. Dunn, unpublished observations).

Mgr1p Is a Mitochondrial IM Protein

MGR1 is predicted to encode a 47.1-kDa protein with two potential membrane-spanning segments. Mgr1p is homologous to proteins encoded by genes found in other yeasts, such as *Kluyveromyces lactis* (KLLA0C01089g; 39% identical by BLASTP), *Eremothecium gossypii* (AFR719W; 32% identical by BLASTP), and the pathogenic *Candida glabrata* (CAGL0B00682g; 38% identical by BLASTP). However, we could not find significant homology to nonfungal proteins. A recent proteomic analysis of yeast mitochondria suggested that Mgr1p is a mitochondrial protein (Sickmann *et al.*, 2003). To confirm this localization, we fused the GFP to the carboxyl-terminus of Mgr1p and expressed this construct in *mgr1*Δ cells. Because Mgr1p-GFP rescued the growth defect of *mgr1*Δ cells grown in EtBr-containing medium, we concluded that the fusion protein is functional (C. Dunn, unpublished observations). When cells expressing Mgr1p-GFP were examined by fluorescence microscopy, we found that the fusion protein was localized to tubular organelles that also stained with the mitochondria-specific dye, Mitofluor 589 (Figure 2A). We conclude that Mgr1p is indeed a mitochondrial protein. Supporting this view, we found that Mgr1p could be imported into isolated mitochondria (Figure 2B). In vitro transcription and translation of *MGR1* produced a ~50-kDa protein. When the ^{35}S-labeled Mgr1 protein was added to energized mitochondria, Mgr1p was imported into the organelle and became resistant to exogenously added protease. When the mitochondrial IM potential was dissipated, however, Mgr1p was not imported and was mostly digested by the protease treatment. In contrast to the presequence-containing su9-DHFR fusion protein, we found the mobility of Mgr1p was not altered after import into mitochondria (Figure 2B). Thus, Mgr1p does not appear to contain a cleavable presequence.

We found that Mgr1p is inserted into the mitochondrial inner membrane. For these analyses, we first inserted 13 tandem myc epitopes (Evan *et al.*, 1985; Longtine *et al.*, 1998) at the carboxyl-terminus of Mgr1p, generating a fully functional protein. When mitochondria isolated from cells expressing Mgr1p-myc were treated with alkali (Figure 2C), both Mgr1p-myc and Tim23p, a protein previously shown to reside within the IM (Emtage and Jensen, 1993), were found in the pellet fraction. In contrast, almost all of Mas2p, a soluble matrix protein (Jensen and Yaffe, 1988) was found in the supernatant fraction after carbonate extraction. Thus, our results indicate that Mgr1p-myc is an integral membrane protein. We also found that Mgr1p is an inner membrane protein. When membrane vesicles from Mgr1p-myc mitochondria were separated on sucrose gradients, Mgr1p-myc cofractionated with the inner membrane marker F1β, and not with the outer membrane protein OM45 (Figure 2D).

We found that the carboxyl-terminus of Mgr1p faces the IMS. When intact mitochondria containing Mgr1p-myc were treated with protease, Mgr1p-myc, along with the inner membrane Tim23 protein, and matrix-localized Mas2p were protected from digestion (Figure 2E). Only the outer membrane Tom70 protein was removed by the protease treatment. In contrast, when the mitochondrial outer membrane was disrupted by osmotic shock (OS) to form mitoplasts, the immunoreactive domain of Tim23p that faces the IMS (Ryan *et al.*, 1998) and the carboxyl-terminal epitope of Mgr1p-myc were now digested. Only the matrix Mas2 protein was protected from the protease in osmotically shocked mitochondria. We note that no new, protease-protected Mgr1p-myc fragments were detected when mitoplasts were treated with protease. Because Mgr1p is predicted to contain two transmembrane segments, our results suggest that Mgr1p resides in the mitochondrial IM with both its amino- and carboxyl-termini facing the IMS.

Mgr1p Is a Subunit of the i-AAA Protease Complex

Our preliminary analysis using gel filtration of detergent-solubilized mitochondria indicated that Mgr1p exists in the IM within a structure of more than 600 kDa (C. Dunn, unpublished observations). Several protein complexes in the IM are important for yeast cell viability in the absence of mtDNA, and three of these complexes, the F_1F_O-ATP synthase, the i-AAA protease, and the prohibitin complex, are larger than 600 kDa (Leonhard *et al.*, 1996; Arnold *et al.*, 1998; Steglich *et al.*, 1999). In coimmunoprecipitation studies, we found that Mgr1p is a new member of the i-AAA protease complex. The i-AAA complex has been shown to be ~850 kDa in size, with only one known subunit, the 82-kDa Yme1 protein (Langer *et al.*, 2001; Arnold and Langer, 2002). We isolated mitochondria from cells expressing Mgr1p-myc and solubilized the mitochondria in buffer containing 1% digitonin. We then immune-precipitated Mgr1p-myc using antibodies to the myc epitope and analyzed the pellet fractions by Western blotting. As shown in Figure 3A, our antibodies pelleted a significant amount of Mgr1p-myc from the mitochondrial lysate. We found that a similar amount of Yme1p coprecipitated with Mgr1p-myc, indicating that Yme1p is associated with the Mgr1 protein in mitochondria. Other IM

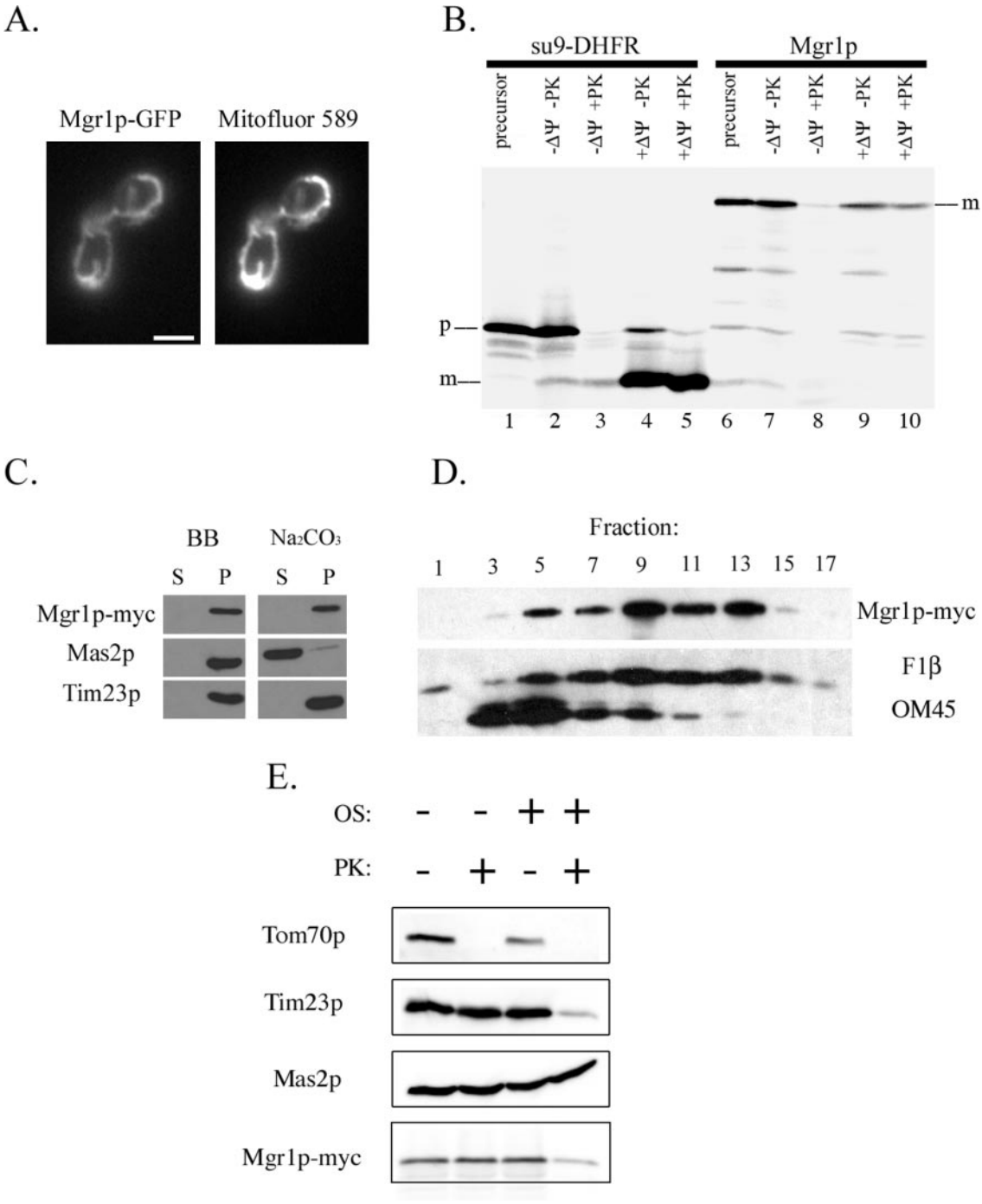

Figure 2. Mgr1p is a mitochondrial IM protein with its carboxyl-terminus facing the IMS. (A) Mgr1p is a mitochondrial protein. *mgr1*Δ strain RJ1688 with plasmid pM486, which expresses the Mgr1p-GFP fusion protein, was stained with 2 μM Mitofluor 589 (Molecular Probes) and examined by fluorescence microscopy. Bar, 2 μm. (B) Mgr1p is imported into isolated mitochondria. ^{35}S-labeled Mgr1p or su9-DHFR were mixed with mitochondria from wild-type strain RJ1962 with (+$\Delta\Psi$) or without (−$\Delta\Psi$) an inner membrane potential. Some samples (+PK) were digested after the import reaction with 100 μg/ml proteinase K on ice for 20 min. After centrifugation, samples were analyzed by SDS-PAGE and phosphorimaging. The precursor (p) and mature (m) forms of su9-DHFR and Mgr1p are indicated. (C) Mgr1p is an integral membrane protein. Mitochondria from strain RJ1970, which expresses Mgr1p-myc, were resuspended in breaking buffer (BB; 0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4) or in 0.1 M sodium carbonate, pH 11. After centrifugation, equal amounts of the supernatant (S) or pellet (P) fractions were analyzed by Western blotting using antibodies to the myc epitope (Mgr1p-myc), Tim23p, an integral membrane protein, or Mas2p, a soluble matrix marker. (D) Mgr1p is located in the mitochondrial IM. Mgr1p-myc mitochondria from strain RJ1970 were sonicated and membrane vesicles separated on sucrose step gradients. Fractions from the gradients were analyzed by Western blotting with antibodies to the myc epitope (Mgr1p-myc), the IM protein F1β, or the OM protein, OM45. Fraction 1 represents the top of the gradient. (E) The carboxyl-terminus of Mgr1p-myc faces the IMS. Intact mitochondria from *MGR1-MYC* strain RJ1970, or mitochondria whose OM was disrupted by osmotic shock (+OS), were digested with 50 μg/ml PK for 30 min on ice and then analyzed by Western blotting using antibodies to the myc epitope (Mgr1p-myc), the matrix protein Mas2p, the OM protein Tom70p, or the IM protein Tim23p.

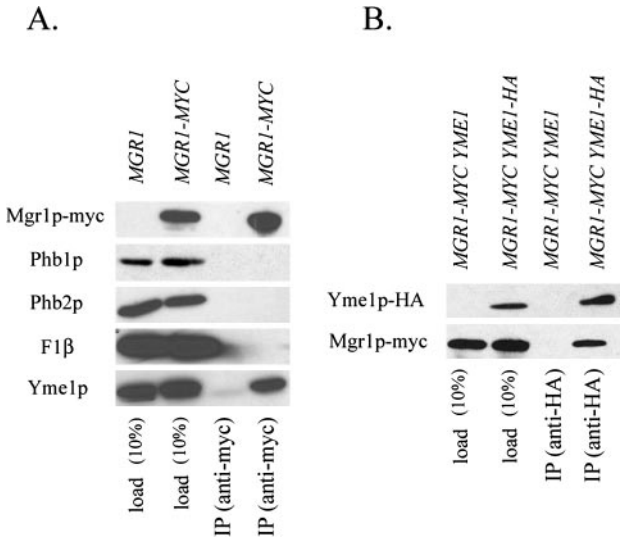

Figure 3. Mgr1p is a subunit of the i-AAA protease complex. (A) Yme1p coprecipitates with Mgr1p-myc. Mitochondria from *MGR1-MYC* strain RJ1970 were solubilized in buffer containing 1% digitonin, and the Mgr1p-myc protein was then precipitated using anti-myc antibodies coupled to agarose beads. Aliquots from the starting lysate (load) and the pellet fraction (IP) were analyzed by Western blotting with antibodies to the myc epitope (Mgr1p-myc), Phb1p, Phb2p, F1β, and Yme1p. As a control, mitochondrial lysates from wild-type mitochondria (*MGR1*) were analyzed in parallel. (B) Mgr1p-myc coprecipitates with Yme1p-HA. Mitochondria isolated from strain RJ1980, expressing both Mgr1p-myc and Yme1p-HA fusion proteins, were solubilized in digitonin-containing buffer, and the Yme1p-HA protein precipitated with agarose beads linked to anti-HA antibodies. As a control, mitochondria from *YME1 MGR1-MYC* strain RJ1970 were also analyzed. Aliquots from the mitochondrial lysate (load) and precipitated proteins (IP) were Western blotted using anti-HA (Yme1p-HA) or anti-myc (Mgr1p-myc) antibodies.

proteins that are not part of the i-AAA complex, such as the β-subunit of the F_1-ATPase or the two prohibitin complex members Phb1p and Phb2p, did not precipitate with Mgr1p-myc. To further investigate the interaction between Yme1p and Mgr1p, we inserted three tandem HA-epitopes (Niman *et al.*, 1983; Longtine *et al.*, 1998) at the carboxyl-terminus of Yme1p in a strain that also expressed Mgr1p-myc. Mitochondria from this strain were solubilized in digitonin buffer, and the Yme1p-HA fusion protein was immunoprecipitated. We found that Mgr1p-myc coimmunoprecipitated along with Yme1p-HA (Figure 3B), further suggesting a physical association between Mgr1p and Yme1p. We note that the Mgr1p-Yme1p interaction is not detergent specific, because Mgr1p and Yme1p also coprecipitated when mitochondria were solubilized with 2% Triton X-100 (C. Dunn, unpublished observations). Furthermore, even though the Yme1 protein is an ATP-dependent protease (Nakai *et al.*, 1995; Leonhard *et al.*, 1996; Weber *et al.*, 1996), the presence or absence of ATP in our buffers made no difference in the Mgr1p-Yme1p interaction (C. Dunn, unpublished observations).

Also suggesting that Mgr1p is part of the Yme1p-containing i-AAA complex, we found that Mgr1p comigrates with Yme1p during blue-native PAGE (BN-PAGE). In these studies, mitochondria containing both Mgr1p-myc and Yme1p-HA were solubilized with digitonin-containing buffer, and membrane complexes were separated on nondenaturing gels. Western blots of our blue-native gels indicated that the majority of Yme1p-HA migrated in a large complex of ~800 kDa (Figure 4A, open arrow), consistent with its previously reported size (Leonhard *et al.*, 1996). Surprisingly, we also found a significant amount of Yme1p-HA in a slightly smaller complex (Figure 4A, black arrow). We found that the bulk of Mgr1p-myc comigrated with the larger form of the Yme1p-containing complex, with a lesser amount of Mgr1p-myc migrating as several smaller species, possibly representing other Mgr1p complexes. We also note that when we attempted to separate Mgr1p-myc, Yme1p, and Yme1p-HA complexes using BN-PAGE, some protein did not enter our resolving gels (Figure 4, A–C, asterisks) and therefore may exist in very large complexes that we cannot resolve using this technique.

Disruption of *MGR1* altered the mobility of the i-AAA protease during BN-PAGE. When mitochondria were isolated from *mgr1*Δ cells, we found that most Yme1p was no longer in the larger complex seen in wild-type mitochondria (Figure 4B; open arrow). Almost all of Yme1p in *mgr1*Δ mitochondria was now found in the smaller form (Figure 4B, black arrow). In contrast to the effect of the *mgr1*Δ mutation on i-AAA protease structure, we saw no effect on Yme1p mobility in mitochondria isolated from a mutant lacking the prohibitin complex (Figure 4B). Similar to the way the Yme1p complex changed in the absence of Mgr1p, the size of the Mgr1p-containing complex depends on Yme1p. When mitochondria were isolated from *yme1*Δ cells and subjected to BN-PAGE, we found that instead of the ~800-kDa complex seen in wild-type mitochondria (Figure 4C, open arrow), Mgr1p-myc migrated mostly in a form of ~400 kDa (Figure 4C, black arrow). Taken together, our coprecipitation and BN-PAGE studies strongly suggest that Mgr1p and Yme1p are both members of the i-AAA complex.

Mitochondria Lacking Mgr1p Are Defective in Protein Turnover

The i-AAA protease complex has been shown to mediate turnover of a number of IM proteins (Nakai *et al.*, 1995; Pearce and Sherman, 1995; Weber *et al.*, 1996; Lemaire *et al.*, 2000; Leonhard *et al.*, 2000; Kominsky *et al.*, 2002; Augustin *et al.*, 2005). One such substrate is Nde1p, subunit 1 of a mitochondrial NADH dehydrogenase complex that has a catalytic domain located in the IMS (Luttik *et al.*, 1998). Fusion of the HA epitope to the carboxyl-terminus of Nde1p creates an unstable protein whose turnover is deficient in *yme1*Δ mitochondria (Augustin *et al.*, 2005). To examine the role of Mgr1p in protein turnover, we generated wild-type, *mgr1*Δ, and *yme1*Δ strains, each expressing the Nde1p-HA construct. We added cycloheximide to cultures of these three strains to stop protein synthesis and then examined the Nde1p-HA levels at different times after the translation arrest. In wild-type cells, Nde1p-HA disappeared with a half-life of ~15 min (Figure 5A). Consistent with previous results (Augustin *et al.*, 2005), we found that Nde1p-HA was much more stable in *yme1*Δ cells. No turnover of Nde1p-HA was seen in the 75 min of our study. Although the proteolysis defect of *mgr1*Δ cells was not as dramatic as that of *yme1*Δ cells, we found that degradation of Nde1p-HA was significantly reduced when Mgr1p was absent. We estimated that the initial half-life of Nde1p-HA in *mgr1*Δ cells is ~23 min, a ~50% increase compared with wild-type cells. We noted that the Nde1p-HA turnover defect in *mgr1*Δ and *yme1*Δ cells was also apparent before cycloheximide treatment. Steady state levels of Nde1p-HA were ~4-fold higher in *yme1*Δ cells and ~1.5-fold higher in *mgr1*Δ cells than in wild-type cells (Figure 5A, see 0-min time points).

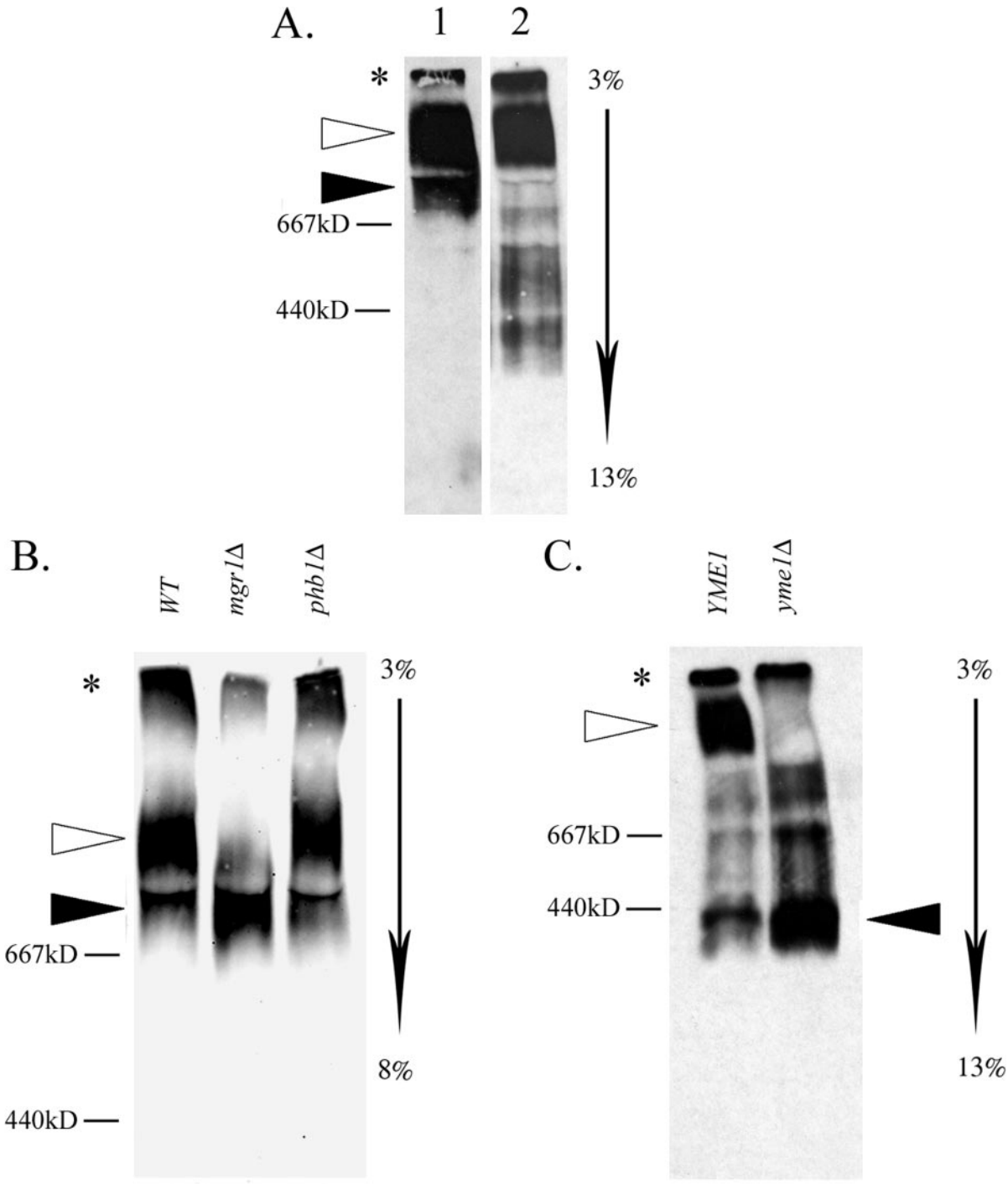

Figure 4. The i-AAA protease complex requires both Mgr1p and Yme1p. (A) Mgr1p comigrates with Yme1p during blue-native gel electrophoresis. Mitochondria isolated from strain RJ1980 were solubilized in digitonin-containing buffer, and protein complexes were separated on a 3–13% polyacrylamide blue-native gel. Western blotting was used to locate the (1) Yme1p-HA and (2) Mgr1p-myc proteins. The open and black arrows denote two apparent forms of the i-AAA complex. (B) The size of the Yme1p-containing complex decreases in the absence of Mgr1p. Mitochondria were isolated from *WT* (RJ1962), *mgr1*Δ (RJ1961), and *phb1*Δ (RJ2083) cells and then solubilized in digitonin buffer. Protein complexes were separated on a 3–8% polyacrylamide blue-native gel and analyzed by Western blotting with anti-Yme1p antibodies. The open and black arrows denote two apparent forms of the i-AAA complex. (C) The mobility of the main Mgr1p-containing complex changes in mitochondria lacking Yme1p. Mitochondria isolated from wild-type cells expressing Mgr1p-myc (RJ1970), or *yme1*Δ cells expressing Mgr1p-myc (RJ1972) were solubilized, run on a 3–13% polyacrylamide blue-native gel and analyzed by Western blotting with anti-myc antibodies. The arrows denote the two main forms of the Mgr1p-containing complex. In A, B, and C, Yme1p, Yme1p-HA, or Mgr1p-myc that has not entered the resolving gel is labeled with an asterisk.

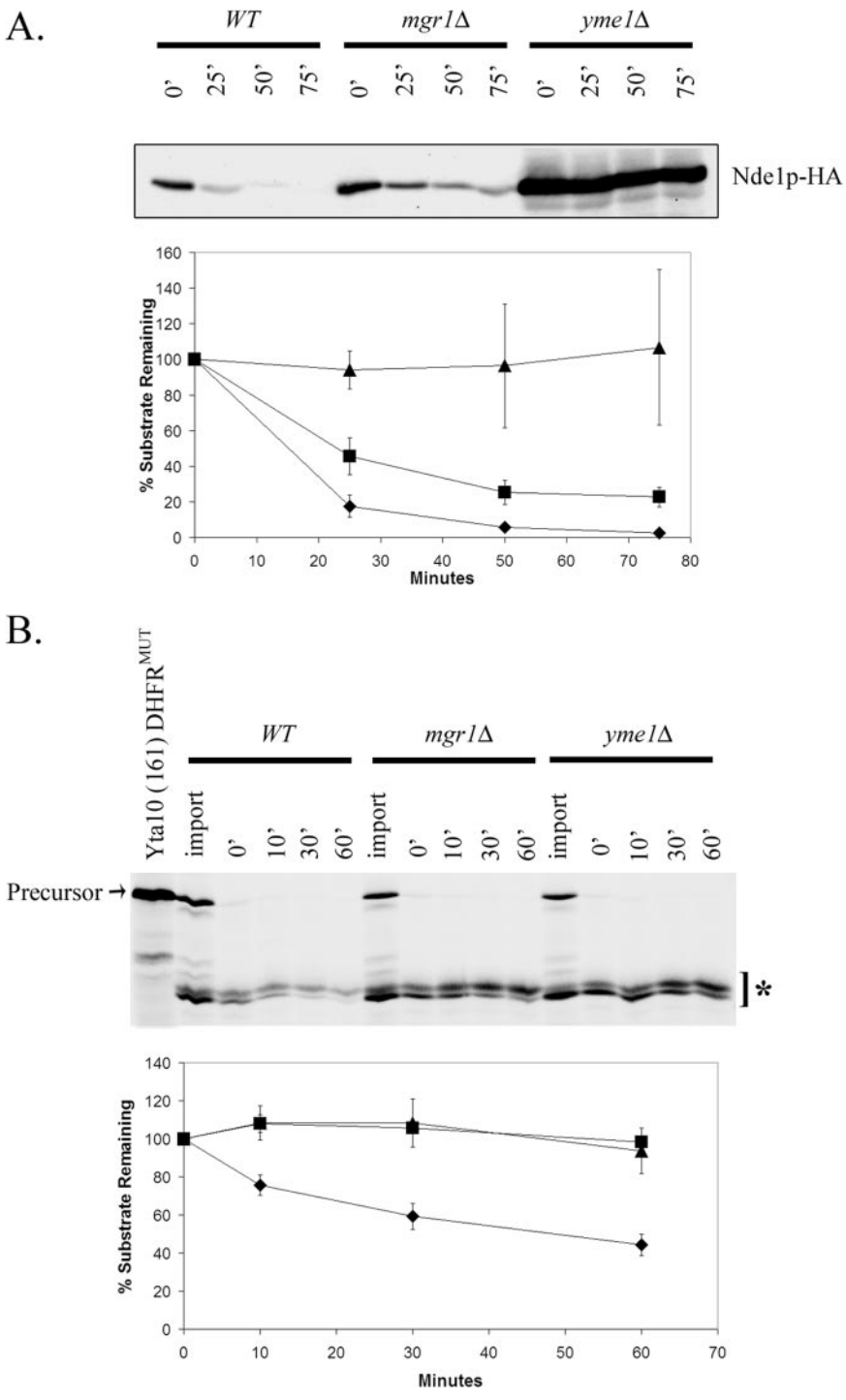

Figure 5. Mitochondria lacking Mgr1p are defective in turnover of IM proteins. (A) The Nde1p-HA fusion protein is more stable in *mgr1*Δ and *yme1*Δ cells. Wild-type strain RJ2077 (diamonds), *mgr1*Δ strain RJ2078 (squares), and *yme1*Δ strain RJ2079 (triangles) each expressing Nde1p-HA were grown to midlog phase at 30°C in YEP glycerol/ethanol medium. Cycloheximide was then added to 1 mg/ml and the cultures were shifted to 37°C. At the indicated times, cells were lysed, equal amounts of cellular protein were loaded in each lane, and the amount of Nde1p-HA was determined by Western blotting using antibodies to the HA-epitope (n = 3). (B) Turnover of the Yta10(161)-DHFR^{MUT} fusion protein is reduced in *mgr1*Δ and *yme1*Δ mitochondria. The ^{35}S-labeled Yta10(161)-DHFR^{MUT} precursor was imported at 25°C for 10 min into mitochondria isolated from *WT* strain RJ1962 (diamonds), *mgr1*Δ strain RJ1961 (squares), and *yme1*Δ strain RJ1974 (triangles). Precursor not imported was digested with 100 μg/ml proteinase K on ice for 30 min. After the addition of 1 mM PMSF, samples were shifted to 25°C and incubated for the indicated times. Equal amounts of mitochondria were isolated by centrifugation at each time point, subjected to SDS-PAGE, and analyzed by phosphorimaging (n = 3). The processed forms of Yta10(161)-DHFR^{MUT} (asterisk) were quantified.

The requirement for Mgr1p in protein turnover was also seen during in vitro assays. Previous studies found that Yta10(161)-DHFR^{MUT}, containing amino acids 1–161 of the inner membrane Yta10 protein fused to a folding mutant version of dihydrofolate reductase (DHFR^{MUT}) is rapidly degraded after import into mitochondria (Leonhard *et al.*, 1999). We imported Yta10(161)-DHFR^{MUT} into mitochondria isolated from wild-type, *mgr1*Δ, and *yme1*Δ cells, removed nonimported precursor by protease treatment, and followed the Yta10(161)-DHFR^{MUT} levels during a 60-min incubation in the presence of ATP. As shown in Figure 5B, the Yta10(161)-DHFR^{MUT} precursor is imported into wild-type, *mgr1*Δ, and *yme1*Δ mitochondria and rapidly converted to a smaller form by the matrix-localized processing protease (Leonhard *et al.*, 1999; Baumann *et al.*, 2002). In contrast to a previous study (Leonhard *et al.*, 1999) that reported a single processed species of Yta10(161)-DHFR^{MUT} after import, we detected two closely spaced bands (Figure 5B, asterisk) of processed Yta10(161)-DHFR^{MUT}. Because the significance of the two forms is not clear, we chose to follow both bands and report the sum of the two forms in our quantifications (Figure 5B). In wild-type mitochondria, imported and pro-

cessed Yta10(161)-DHFR^{MUT} disappeared with an initial half-life of ~23 min. In *yme1*Δ mitochondria, the processed Yta10(161)-DHFR^{MUT} persisted and no significant turnover was detected. We found that the defect in degradation of Yta10(161)-DHFR^{MUT} in *mgr1*Δ mitochondria was similar to that of *yme1*Δ mitochondria, in that there was no appreciable degradation of imported substrate in *mgr1*Δ mitochondria. Our results using two different i-AAA protease substrates demonstrate a requirement for Mgr1p in the efficient degradation of misfolded substrates.

*mgr1*Δ *Mutants Show Less Severe Phenotypes than yme1*Δ *Cells*

In addition to its petite-negative phenotype, the *yme1*Δ mutant has several other defects. For example, *yme1* was initially isolated due to its increased rate of mtDNA escape to the nucleus (Thorsness and Fox, 1993). Moreover, *yme1*Δ cells cannot grow on glycerol/ethanol medium at high temperature and are cold-sensitive on glucose medium (Thorsness and Fox, 1993). We find that although Mgr1p is part of the i-AAA complex along with Yme1p, the *mgr1*Δ mutant does not exhibit any of the additional properties of *yme1*Δ cells. Loss of Mgr1p does not increase mtDNA escape (Figure 6A) and *mgr1*Δ cells are not heat- (Figure 6B) or cold-sensitive (Figure 6C) for growth. Because *mgr1*Δ cells show fewer defects than *yme1*Δ cells, we conclude that there is not a complete loss of i-AAA protease function in the *mgr1*Δ mutant. We have found that *mgr1*Δ *yme1*Δ double mutants do not exhibit synthetic lethality or any other detectable synthetic growth phenotype (C. Dunn, unpublished observations). Because the double mutant is indistinguishable from the *yme1*Δ mutant, we argue that the role of Mgr1p may be restricted to its interaction with Yme1p in the i-AAA complex.

Generation of Yeast Cells Lacking Both the m-AAA and i-AAA Protease Complexes

The mitochondrial IM contains two AAA protease complexes, the i-AAA protease, whose catalytic domain faces the IMS, and the m-AAA protease, which faces the matrix (Langer *et al.*, 2001; Arnold and Langer, 2002). Previous studies suggest that the two assemblies might overlap in their substrate selection (Leonhard *et al.*, 2000) and work together to degrade one or more mitochondrial proteins. Consistent with this idea, yeast mutants deficient in either the i-AAA or the m-AAA protease complex are viable, but double mutants lacking both complexes are dead (Lemaire *et al.*, 2000; Leonhard *et al.*, 2000). However, the petite-negative phenotype of the *yme1*Δ mutant suggests an alternative explanation for the lethality of cells lacking both i-AAA and m-AAA protease complexes. *yta10*Δ and *yta12*Δ cells, which lack m-AAA activity, behave like petite mutants—they grow very poorly on nonfermentable medium (Guelin *et al.*, 1994; Tauer *et al.*, 1994; Tzagoloff *et al.*, 1994). This petite phenotype of *yta10*Δ and *yta12*Δ mutants is thought to result from defects in the assembly of inner membrane protein complexes (Tzagoloff *et al.*, 1994; Paul and Tzagoloff, 1995). We postulated that the petite phenotype of *yta10*Δ and *yta12*Δ mutants could be incompatible with the petite-negative *yme1*Δ mutant. To test this idea, we constructed a diploid strain heterozygous for disruptions in *YTA10, YTA12,* and *YME1*. To suppress the petite-negative defect of the *yme1*Δ mutant (but not the i-AAA-dependent proteolysis defect), we transformed the diploid with a plasmid carrying the *ATP3-5* allele. *ATP3-5* encodes a mutant version of the γ-subunit of the F_1-ATPase and allows *yme1*Δ cells to grow without mtDNA (Weber *et al.*, 1995). As a control, a plasmid encoding the wild-type *ATP3* allele was also used. After sporulation, 16 tetrads from each diploid were dissected onto YEPD medium and incubated for 5 d. As expected, diploids transformed with the wild-type *ATP3* gene yielded no viable colonies composed of *yme1*Δ *yta10*Δ, *yme1*Δ *yta12*Δ, or *yme1*Δ *yta10*Δ *yta12*Δ cells. In contrast, 7 *yme1*Δ *yta10*Δ or *yme1*Δ *yta12*Δ double mutants, and six triple mutants were isolated from diploids carrying the *ATP3-5* plasmid. Our results thus indicate that yeast cells lacking both m-AAA and i-AAA protease activity can be constructed, and that the synthetic lethality of *yme1*Δ *yta10*Δ and *yme1*Δ *yta12*Δ double mutants is due to the petite-negative property of the *yme1*Δ mutant and perhaps not due simply to overlapping substrate selection of the i-AAA and m-AAA proteases. We speculate that other examples of synthetic lethality, such as for mutants lacking both the prohibitin complex and genes required for mitochondrial shape (Berger and Yaffe, 1998), or for mutants lacking both m-AAA protease and prohibitin function (Steglich *et al.*, 1999), might instead be due to the intolerable combination of a mutation that confers mtDNA-dependence, such as *phb1*Δ (see Figure 1), with a mutation that causes cells to either lose their mtDNA or to lose the ability to properly assemble mtDNA-encoded proteins into

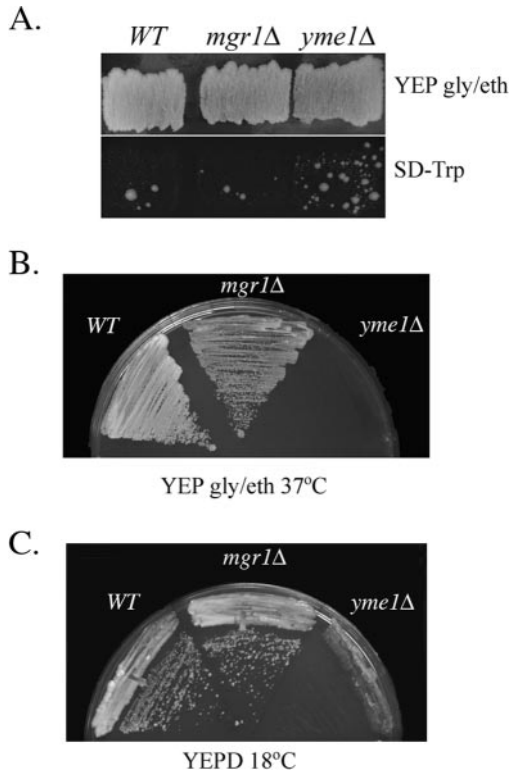

Figure 6. Several phenotypes of the *yme1*Δ mutant are not found in *mgr1*Δ cells. (A) Lack of Mgr1p does not increase mtDNA escape to the nucleus. *trp1* strain PTY44, *trp1 mgr1*Δ strain RJ2001, and *trp1 yme1*Δ strain RJ2040, each containing the nuclear *TRP1* marker in their mitochondria, were grown on YEP glycerol/ethanol medium for 4 d at 30°C. Strains were then replica-plated to SD medium lacking tryptophan and grown for a further 4 d at 30°C. (B) *mgr1*Δ mutants are not heat-sensitive for growth. *WT* (PTY44), *mgr1*Δ (RJ2001), and *yme1*Δ (RJ2040) strains were struck to YEP glycerol/ethanol medium and incubated at 37°C for 5 d. (C) *mgr1*Δ cells are not cold-sensitive. *WT* (BY4742), *mgr1*Δ (RJ2059), and *yme1*Δ (RJ2051) strains were struck to YEPD medium and incubated for 6 d at 18°C.

functional complexes. Regardless, we have shown that viable yeast cells lacking both i-AAA and m-AAA activities can be constructed.

DISCUSSION

In contrast to many eukaryotic organisms, *S. cerevisiae* is able to grow in the absence of mtDNA. In an attempt to better understand the molecular basis for this survival, we conducted a large-scale hunt for yeast mutants that are dependent on their mtDNA for cell viability. Using a microarray-based approach, we searched among viable YKOs for those unable to grow when mtDNA loss was induced by growth on EtBr-containing medium. Although we have identified several new genes, it is clear that our screen was not exhaustive. Our microarray analysis yielded some previously known mutants, such as *fmc1*Δ, which are defective in assembly of the F_1 sector of the mitochondrial F_1F_O-ATP synthase (Lefebvre-Legendre *et al.,* 2001), and *tim18*Δ and *tom70*Δ (Kerscher *et al.,* 2000; Dunn and Jensen, 2003), which are defective in protein import. Other known petite-negative mutants were not found. These include mutants lacking subunits of the F_1-ATPase, *atp1*Δ, *atp2*Δ, and *atp3*Δ, and the *yme1*Δ mutant, defective in the i-AAA protease complex. We know that many strains were missing from the pool of YKOs that we screened. Only ~3800 out of the total ~4900 viable YKOs were detected in the final pools. This is likely a byproduct of the progressive loss of slow growing mutants during the 18 generations of outgrowth. Moreover, some of YKO strains contain tag sequence mutations preventing proper hybridization of the probe to the microarray (Eason *et al.,* 2004). Although there may be additional genes to be identified through modifications of procedure, this study adds many interesting new proteins to the inventory.

Among the mutants that we identified in our screen for yeast knockouts that cannot live without mtDNA was a strain disrupted in an uncharacterized ORF (*YCL044C*), encoding a protein that we have named Mgr1p. We found that Mgr1p, like the Yme1 protein, is a subunit of the i-AAA protease complex in the mitochondrial IM. We also find that *mgr1*Δ mutants, like *yme1*Δ cells, are defective in the turnover of IM proteins. However, because the Mgr1 protein contains no conserved protease motifs, we speculate that Mgr1p regulates the import, assembly or activity of the catalytic subunit, Yme1p. Suggesting a role in assembly, we found that in wild-type cells Yme1p is located in two large IM complexes with masses of ~700 and ~800 kDa, but in *mgr1*Δ cells, the Yme1 protein is shifted to the smaller, ~700-kDa form. Alternatively, the Mgr1 protein may function like *Escherichia coli* SspB, which delivers substrates to the ClpXP complex, a bacterial member of the AAA protease family (Levchenko *et al.,* 2000). Because the most conserved region of Mgr1p (amino acids 37–53) is localized to the IMS, the Mgr1 protein is poised to present unfolded or unassembled IM proteins to the IMS-facing protease domain of Yme1p (Leonhard *et al.,* 1996).

Why are the *yme1*Δ and *mgr1*Δ mutants dependent on mtDNA for viability? In rho^0 cells, both the electron transport chain and the F_O portion of the ATP synthase are not functional because of the lack of several mtDNA-encoded subunits (Tzagoloff, 1982). Therefore, the essential mitochondrial potential can be generated by neither electron transport chain activity nor reversal of the ATP synthase (i.e., turning the ATPase into a proton pump). However, the F_1 part of the ATP synthase remains active in petite cells and appears critical in generating the IM potential (Ebner and Schatz, 1973; Giraud and Velours, 1997; Chen and Clark-Walker, 1999). ATP made by glycolysis in the cytosol is transported into mitochondria via the inner membrane ATP/ADP carrier proteins in exchange for the ADP produced by the matrix-localized F_1-ATPase. The i-AAA protease is proposed to play an important role in regulating F_1-ATPase activity in petite cells. For example, mitochondria isolated from *yme1*Δ cells depleted of mtDNA contain reduced F_1-ATPase activity and are deficient in generating $\Delta\Psi$ with added ATP (Kominsky *et al.,* 2002). Moreover, the need for Yme1p can be bypassed by specific mutations in F_1-ATPase subunits (Weber *et al.,* 1995). One hypothesis for the requirement for i-AAA protease activity in petite cells is that the Yme1p-containing complex degrades an inhibitor of the F_1-ATPase. Supporting this idea, deletion of the F_1-ATPase inhibitor, Inh1p, bypasses the need for Yme1p in petite cells (Kominsky *et al.,* 2002). However, because the Inh1 protein is located in the matrix and the catalytic domain of i-AAA is in the IMS, it is unlikely that Inh1p is a direct target of the i-AAA protease.

An alternative explanation for the mtDNA-dependence of *yme1*Δ and *mgr1*Δ mutants is that the i-AAA protease is needed to degrade the unassembled, nuclear-encoded subunits of the oxidative phosphorylation machinery that accumulate in the absence of the mtDNA-encoded subunits. Supporting a requirement for robust IM quality control in rho^0 cells, we found that the *phb1*Δ mutant, deficient in a putative IM chaperone (Nijtmans *et al.,* 2000), is petite-negative. Accumulation and possible aggregation of misassembled proteins in *mgr1*Δ, *yme1*Δ, and *phb1*Δ mutants could lead to "leaks" in the IM and overwhelm the ability of the F_1-ATPase to maintain $\Delta\Psi$ in rho^0 mitochondria. In this model, the suppression of petite-negative phenotype of the *yme1*Δ mutant by disrupting *INH1* can be explained by the observation that F_1-ATPase inhibitors like Inh1p are more potent when the IM potential is decreased (Ichikawa *et al.,* 1990; Walker, 1994). Further studies are clearly needed to clarify the requirement for the i-AAA protease in cells lacking mtDNA.

In addition to a new i-AAA complex member, our screen also identified YKOs lacking known proteins with no previous connection to mtDNA dependence. These proteins, which include Thr1p, Thr4p, Ira2p, Pde2p, and Opi1p, play roles in a surprising number of different cellular processes. Thr1p and Thr4p are involved in threonine biosynthesis (Mannhaupt *et al.,* 1990; Ramos and Calderon, 1994). Ira2p, a Ras-GAP protein (Tanaka *et al.,* 1990), and Pde2p, a cyclical AMP phosphodiesterase (Sass *et al.,* 1986), are components of the protein kinase A (PKA) signal transduction pathway. Opi1p is a regulatory protein, repressing the transcription of phospholipid biosynthetic enzymes (Greenberg *et al.,* 1982). Although it is unexpected that these proteins were identified in our screen, some of them have clear links to mitochondrial function. For example, several mutants auxotrophic for threonine were previously found to be deficient in cytochrome oxidase activity (Robichon-Szulmajster *et al.,* 1969; Surdin *et al.,* 1969), and the PKA pathway controls the expression of at least one mitochondrial chaperone (Demlow and Fox, 2003). Even though Thr1p, Thr4p, Ira2p, Pde2p, and Opi1p play such diverse roles, we favor the possibility that they all might affect a common process, such as the generation of the IM potential in cells lacking mtDNA. For example, these proteins might affect the expression, import, assembly, or activity of the F_1-ATPase or the ATP/ADP carrier proteins. Supporting this possibility, we find that a multicopy suppressor that rescues the mtDNA dependence

of petite-negative strains such as *tim18*Δ and *atp2* (Dunn and Jensen, 2003) also suppresses the mtDNA dependence of *mgr2*Δ, *ira2*Δ, *pde2*Δ, and *opi1*Δ mutants (C. Dunn, unpublished observations).

Our studies yielded at least one uncharacterized gene besides *MGR1* that is required by cells lacking mtDNA. One of these new genes, *YPL098C*, which we have named *MGR2*, is listed in Supplementary Table 3 and encodes a 12-kDa integral membrane protein. Our microarray analysis also identified several other novel mutants whose growth was curtailed by the loss of mtDNA, but because these strains exhibited a modest growth defect when directly tested on EtBr medium they were not included in our final list. Nevertheless, it is likely that several of these mutants are likely to carry out significant functions in petite cells and will provide new clues about the requirement for the mitochondrial genome.

ACKNOWLEDGMENTS

We thank Hiromi Sesaki, Kara Cerveny, Matt Youngman, Lee Kapp, and Gulayse Ince Dunn for critical comments on the manuscript and Seth Studer and Sheryl Southard for excellent technical assistance. We also thank Dr. Thomas Langer for the Yta10(161)-DHFR^{MUT} plasmid and Phb1p and Phb2p antiserum, Dr. Peter Thorsness for strains and Yme1p antiserum, Alison Davis for the F1β antiserum, and Jef Boeke for use of microarray slides. This work was supported by National Institutes of Health Grants GM46803 to R.E.J., GM62368 and HG02432 to F.S., and an NIH Predoctoral Training Grant 5T32GM07445 to C.D.D.

REFERENCES

Adams, A., Gottschling, D., Kaiser, C., and Stearns, T. (1997). Methods in Yeast Genetics, Plainview, NY: Cold Spring Harbor Laboratory Press.

Arnold, I., and Langer, T. (2002). Membrane protein degradation by AAA proteases in mitochondria. Biochim. Biophys. Acta *1592*, 89–96.

Arnold, I., Pfeiffer, K., Neupert, W., Stuart, R. A., and Schagger, H. (1998). Yeast mitochondrial F1F0-ATP synthase exists as a dimer: identification of three dimer-specific subunits. EMBO J. *17*, 7170–7178.

Attardi, G., and Schatz, G. (1988). Biogenesis of mitochondria. Annu. Rev. Cell Biol. *4*, 289–333.

Augustin, S., Nolden, M., Muller, S., Hardt, O., Arnold, I., and Langer, T. (2005). Characterization of peptides released from mitochondria: evidence for constant proteolysis and peptide efflux. J. Biol. Chem. *280*, 2691–2699.

Baumann, F., Neupert, W., and Herrmann, J.M. (2002). Insertion of bitopic membrane proteins into the inner membrane of mitochondria involves an export step from the matrix. J. Biol. Chem. *277*, 21405–21413.

Berger, K. H., and Yaffe, M. P. (1998). Prohibitin family members interact genetically with mitochondrial inheritance components in *Saccharomyces cerevisiae.* Mol. Cell. Biol. *18*, 4043–4052.

Bolstad, B. M., Irizarry, R. A., Astrand, M., and Speed, T. P. (2003). A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics *19*, 185–193.

Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P., and Boeke, J. D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115–132.

Chen, X. J., and Clark-Walker, G. D. (1999). Alpha and beta subunits of F1-ATPase are required for survival of petite mutants in *Saccharomyces cerevisiae.* Mol. Gen. Genet *262*, 898–908.

Chen, X. J., and Clark-Walker, G. D. (2000). The petite mutation in yeasts: 50 years on. Int. Rev. Cytol. *194*, 197–238.

Daum, G., Bohni, P. C., and Schatz, G. (1982). Import of proteins into mitochondria. Cytochrome b2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria. J. Biol. Chem. *257*, 13028–13033.

Demlow, C. M., and Fox, T. D. (2003). Activity of mitochondrially synthesized reporter proteins is lower than that of imported proteins and is increased by lowering cAMP in glucose-grown *Saccharomyces cerevisiae* cells. Genetics *165*, 961–974.

Dunn, C. D., and Jensen, R. E. (2003). Suppression of a defect in mitochondrial protein import identifies cytosolic proteins required for viability of yeast cells lacking mitochondrial DNA. Genetics *165*, 35–45.

Dupont, C. H., Mazat, J. P., and Guerin, B. (1985). The role of adenine nucleotide translocation in the energization of the inner membrane of mitochondria isolated from rho + and rho degree strains of *Saccharomyces cerevisiae.* Biochem. Biophys. Res. Commun. *132*, 1116–1123.

Eason, R. G., Pourmand, N., Tongprasit, W., Herman, Z. S., Anthony, K., Jejelowo, O., Davis, R. W., and Stolc, V. (2004). Characterization of synthetic DNA bar codes in *Saccharomyces cerevisiae* gene-deletion strains. Proc. Natl. Acad. Sci. USA *101*, 11046–11051.

Ebner, E., and Schatz, G. (1973). Mitochondrial assembly in respiration-deficient mutants of *Saccharomyces cerevisiae.* 3. A nuclear mutant lacking mitochondrial adenosine triphosphatase. J. Biol. Chem. *248*, 5379–5384.

Emtage, J. L., and Jensen, R. E. (1993). MAS6 encodes an essential inner membrane component of the yeast mitochondrial protein import pathway. J. Cell Biol. *122*, 1003–1012.

Evan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Mol. Cell. Biol. *5*, 3610–3616.

Giaever, G. *et al.* (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. Nature *418*, 387–391.

Giraud, M. F., and Velours, J. (1997). The absence of the mitochondrial ATP synthase delta subunit promotes a slow growth phenotype of rho- yeast cells by a lack of assembly of the catalytic sector F1. Eur. J. Biochem. *245*, 813–818.

Goldring, E. S., Grossman, L. I., Krupnick, D., Cryer, D. R., and Marmur, J. (1970). The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during induction of petites with ethidium bromide. J. Mol. Biol. *52*, 323–335.

Green, D. R., and Kroemer, G. (2004). The pathophysiology of mitochondrial cell death. Science *305*, 626–629.

Greenberg, M. L., Reiner, B., and Henry, S. A. (1982). Regulatory mutations of inositol biosynthesis in yeast: isolation of inositol-excreting mutants. Genetics *100*, 19–33.

Grohmann, L., Graack, H. R., Kruft, V., Choli, T., Goldschmidt-Reisin, S., and Kitakawa, M. (1991). Extended N-terminal sequencing of proteins of the large ribosomal subunit from yeast mitochondria. FEBS Lett. *284*, 51–56.

Guelin, E., Rep, M., and Grivell, L. A. (1994). Sequence of the AFG3 gene encoding a new member of the FtsH/Yme1/Tma subfamily of the AAA-protein family. Yeast *10*, 1389–1394.

Hermann, G. J., Thatcher, J. W., Mills, J. P., Hales, K. G., Fuller, M. T., Nunnari, J., and Shaw, J. M. (1998). Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. J. Cell Biol. *143*, 359–373.

Hoffman, C. S., and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli.* Gene *57*, 267–272.

Ichikawa, N., Yoshida, Y., Hashimoto, T., Ogasawara, N., Yoshikawa, H., Imamoto, F., and Tagawa, K. (1990). Activation of ATP hydrolysis by an uncoupler in mutant mitochondria lacking an intrinsic ATPase inhibitor in yeast. J. Biol. Chem. *265*, 6274–6278.

Jensen, R. E., Dunn, C. D., Youngman, M. J., and Sesaki, H. (2004). Mitochondrial building blocks. Trends Cell Biol. *14*, 215–218.

Jensen, R. E., and Yaffe, M. P. (1988). Import of proteins into yeast mitochondria: the nuclear MAS2 gene encodes a component of the processing protease that is homologous to the MAS1-encoded subunit. EMBO J. *7*, 3863–3871.

Kerscher, O., Sepuri, N. B., and Jensen, R. E. (2000). Tim18p is a new component of the Tim54p-Tim22p translocon in the mitochondrial inner membrane. Mol. Biol. Cell *11*, 103–116.

Kominsky, D. J., Brownson, M. P., Updike, D. L., and Thorsness, P. E. (2002). Genetic and biochemical basis for viability of yeast lacking mitochondrial genomes. Genetics *162*, 1595–1604.

Kovacova, V., Irmlerova, J., and Kovac, L. (1968). Oxidative phosphorylation in yeast. IV. Combination of a nuclear mutation affecting oxidative phosphorylation with cytoplasmic mutation to respiratory deficiency. Biochim. Biophys. Acta *162*, 157–163.

Langer, T., Kaser, M., Klanner, C., and Leonhard, K. (2001). AAA proteases of mitochondria: quality control of membrane proteins and regulatory functions during mitochondrial biogenesis. Biochem. Soc. Trans. *29*, 431–436.

Lee, M. S., and Spencer, F. A. (2004). Bipolar orientation of chromosomes in *Saccharomyces cerevisiae* is monitored by Mad1 and Mad2, but not by Mad3. Proc. Natl. Acad. Sci. USA *101*, 10655–10660.

Lefebvre-Legendre, L., Vaillier, J., Benabdelhak, H., Velours, J., Slonimski, P. P., and di Rago, J. P. (2001). Identification of a nuclear gene (FMC1) required for the assembly/stability of yeast mitochondrial F(1)-ATPase in heat stress conditions. J. Biol. Chem. *276*, 6789–6796.

Lemaire, C., Hamel, P., Velours, J., and Dujardin, G. (2000). Absence of the mitochondrial AAA protease Yme1p restores F0-ATPase subunit accumulation in an oxa1 deletion mutant of *Saccharomyces cerevisiae.* J. Biol. Chem. *275*, 23471–23475.

Leonhard, K., Guiard, B., Pellecchia, G., Tzagoloff, A., Neupert, W., and Langer, T. (2000). Membrane protein degradation by AAA proteases in mitochondria: extraction of substrates from either membrane surface. Mol. Cell *5*, 629–638.

Leonhard, K., Herrmann, J. M., Stuart, R. A., Mannhaupt, G., Neupert, W., and Langer, T. (1996). AAA proteases with catalytic sites on opposite membrane surfaces comprise a proteolytic system for the ATP-dependent degradation of inner membrane proteins in mitochondria. EMBO J. *15*, 4218–4229.

Leonhard, K., Stiegler, A., Neupert, W., and Langer, T. (1999). Chaperone-like activity of the AAA domain of the yeast Yme1 AAA protease. Nature *398*, 348–351.

Levchenko, I., Seidel, M., Sauer, R. T., and Baker, T. A. (2000). A specificity-enhancing factor for the ClpXP degradation machine. Science *289*, 2354–2356.

Longtine, M. S., McKenzie, A., 3rd, Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P., and Pringle, J. R. (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae.* Yeast *14*, 953–961.

Luttik, M. A., Overkamp, K. M., Kotter, P., de Vries, S., van Dijken, J. P., and Pronk, J. T. (1998). The *Saccharomyces cerevisiae* NDE1 and NDE2 genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. J. Biol. Chem. *273*, 24529–24534.

Mannhaupt, G., Pohlenz, H. D., Seefluth, A. K., Pilz, U., and Feldmann, H. (1990). Yeast homoserine kinase. Characteristics of the corresponding gene, THR1, and the purified enzyme, and evolutionary relationships with other enzymes of threonine metabolism. Eur. J. Biochem. *191*, 115–122.

Mannhaupt, G., Schnall, R., Karpov, V., Vetter, I., and Feldmann, H. (1999). Rpn4p acts as a transcription factor by binding to PACE, a nonamer box found upstream of 26S proteasomal and other genes in yeast. FEBS Lett. *450*, 27–34.

Mootha, V. K. *et al.* (2003). Integrated analysis of protein composition, tissue diversity, and gene regulation in mouse mitochondria. Cell *115*, 629–640.

Nakai, T., Yasuhara, T., Fujiki, Y., and Ohashi, A. (1995). Multiple genes, including a member of the AAA family, are essential for degradation of unassembled subunit 2 of cytochrome c oxidase in yeast mitochondria. Mol. Cell. Biol. *15*, 4441–4452.

Nijtmans, L. G., de Jong, L., Artal Sanz, M., Coates, P. J., Berden, J. A., Back, J. W., Muijsers, A. O., van der Spek, H., and Grivell, L. A. (2000). Prohibitins act as a membrane-bound chaperone for the stabilization of mitochondrial proteins. EMBO J. *19*, 2444–2451.

Niman, H. L., Houghten, R. A., Walker, L. E., Reisfeld, R. A., Wilson, I. A., Hogle, J. M., and Lerner, R. A. (1983). Generation of protein-reactive antibodies by short peptides is an event of high frequency: implications for the structural basis of immune recognition. Proc. Natl. Acad. Sci. USA *80*, 4949–4953.

Ooi, S. L., Shoemaker, D. D., and Boeke, J. D. (2001). A DNA microarray-based genetic screen for nonhomologous end-joining mutants in *Saccharomyces cerevisiae.* Science *294*, 2552–2556.

Pan, C., and Mason, T. L. (1995). Identification of the yeast nuclear gene for the mitochondrial homologue of bacterial ribosomal protein L16. Nucleic Acids Res. *23*, 3673–3677.

Paul, M. F., and Tzagoloff, A. (1995). Mutations in RCA1 and AFG3 inhibit F1-ATPase assembly in *Saccharomyces cerevisiae.* FEBS Lett. *373*, 66–70.

Pearce, D. A., and Sherman, F. (1995). Degradation of cytochrome oxidase subunits in mutants of yeast lacking cytochrome c and suppression of the degradation by mutation of yme1. J. Biol. Chem. *270*, 20879–20882.

Pfanner, N., Muller, H. K., Harmey, M. A., and Neupert, W. (1987). Mitochondrial protein import: involvement of the mature part of a cleavable precursor protein in the binding to receptor sites. EMBO J. *6*, 3449–3454.

Pon, L., Moll, T., Vestweber, D., Marshallsay, B., and Schatz, G. (1989). Protein import into mitochondria: ATP-dependent protein translocation activity in a submitochondrial fraction enriched in membrane contact sites and specific proteins. J. Cell Biol. *109*, 2603–2616.

Ramos, C., and Calderon, I. L. (1994). Biochemical evidence that the *Saccharomyces cerevisiae* THR4 gene encodes threonine synthetase. FEBS Lett. *351*, 357–359.

Rapaport, D., Brunner, M., Neupert, W., and Westermann, B. (1998). Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in *Saccharomyces cerevisiae.* J. Biol. Chem. *273*, 20150–20155.

Rehling, P., Brandner, K., and Pfanner, N. (2004). Mitochondrial import and the twin-pore translocase. Nat. Rev. Mol. Cell. Biol. *5*, 519–530.

Robichon-Szulmajster, H., Surdin, Y., and Slonimski, P. P. (1969). Deficiency of cytochrome oxidase in *Saccharomyces cerevisiae* strains requiring threonine for growth. 2. Metabolic relationships. Eur. J. Biochem. *7*, 531–536.

Ryan, K. R., Leung, R. S., and Jensen, R. E. (1998). Characterization of the mitochondrial inner membrane translocase complex: the Tim23p hydrophobic domain interacts with Tim17p but not with other Tim23p molecules. Mol. Cell. Biol. *18*, 178–187.

Sass, P., Field, J., Nikawa, J., Toda, T., and Wigler, M. (1986). Cloning and characterization of the high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae.* Proc. Natl. Acad. Sci. USA *83*, 9303–9307.

Schagger, H., and von Jagow, G. (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. *199*, 223–231.

Senapin, S., Chen, X. J., and Clark-Walker, G. D. (2003). Transcription of TIM9, a new factor required for the petite-positive phenotype of *Saccharomyces cerevisiae*, is defective in spt7 mutants. Curr. Genet. *44*, 202–210.

Sesaki, H., and Jensen, R. E. (1999). Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. J. Cell Biol. *147*, 699–706.

Sesaki, H., Southard, S. M., Yaffe, M. P., and Jensen, R. E. (2003). Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane. Mol. Biol. Cell *14*, 2342–2356.

Shoemaker, D. D., Lashkari, D. A., Morris, D., Mittmann, M., and Davis, R. W. (1996). Quantitative phenotypic analysis of yeast deletion mutants using a highly parallel molecular bar-coding strategy. Nat. Genet. *14*, 450–456.

Sickmann, A. *et al.* (2003). The proteome of *Saccharomyces cerevisiae* mitochondria. Proc. Natl. Acad. Sci. USA *100*, 13207–13212.

Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae.* Genetics *122*, 19–27.

Slonimski, P. P., Perrodin, G., and Croft, J. H. (1968). Ethidium bromide induced mutation of yeast mitochondria: complete transformation of cells into respiratory deficient non-chromosomal "petites." Biochem. Biophys. Res. Commun. *30*, 232–239.

Steglich, G., Neupert, W., and Langer, T. (1999). Prohibitins regulate membrane protein degradation by the m-AAA protease in mitochondria. Mol. Cell. Biol. *19*, 3435–3442.

Surdin, Y., Robichon-Szulmajster, H., Lachowicz, T. M., and Slonimski, P. P. (1969). Deficiency of cytochrome oxidase in *Saccharomyces cerevisiae* strains requiring threonine for growth. 1. Genetic relationships. Eur. J. Biochem. *7*, 526–530.

Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M. S., Gibbs, J. B., Matsumoto, K., Kaziro, Y., and Toh-e, A. (1990). *S. cerevisiae* genes IRA1 and IRA2 encode proteins that may be functionally equivalent to mammalian ras GTPase activating protein. Cell *60*, 803–807.

Tauer, R., Mannhaupt, G., Schnall, R., Pajic, A., Langer, T., and Feldmann, H. (1994). Yta10p, a member of a novel ATPase family in yeast, is essential for mitochondrial function. FEBS Lett. *353*, 197–200.

Thorsness, P. E., and Fox, T. D. (1993). Nuclear mutations in *Saccharomyces cerevisiae* that affect the escape of DNA from mitochondria to the nucleus. Genetics *134*, 21–28.

Thorsness, P. E., White, K. H., and Fox, T. D. (1993). Inactivation of YME1, a member of the ftsH-SEC18-PAS1-CDC48 family of putative ATPase-encoding genes, causes increased escape of DNA from mitochondria in *Saccharomyces cerevisiae.* Mol. Cell. Biol. *13*, 5418–5426.

Trifunovic, A. *et al.* (2004). Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature *429*, 417–423.

Tzagoloff, A. (1982). Mitochondria, New York: Plenum Press.

Tzagoloff, A., Yue, J., Jang, J., and Paul, M. F. (1994). A new member of a family of ATPases is essential for assembly of mitochondrial respiratory chain and ATP synthetase complexes in *Saccharomyces cerevisiae.* J. Biol. Chem. *269*, 26144–26151.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae.* Yeast *10*, 1793–1808.

Walker, J. E. (1994). The regulation of catalysis in ATP synthase. Curr. Opin. Struct. Biol. *4*, 912–918.

Weber, E. R., Hanekamp, T., and Thorsness, P. E. (1996). Biochemical and functional analysis of the YME1 gene product, an ATP and zinc-dependent mitochondrial protease from *S. cerevisiae.* Mol. Biol. Cell *7*, 307–317.

Weber, E. R., Rooks, R. S., Shafer, K. S., Chase, J. W., and Thorsness, P. E. (1995). Mutations in the mitochondrial ATP synthase gamma subunit suppress a slow-growth phenotype of yme1 yeast lacking mitochondrial DNA. Genetics *140*, 435–442.

Winston, F., Dollard, C., and Ricupero-Hovasse, S. L. (1995). Construction of a set of convenient *Saccharomyces cerevisiae* strains that are isogenic to S288C. Yeast *11*, 53–55.

Winzeler, E. A. *et al.* (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science *285*, 901–906.

Xie, Y., and Varshavsky, A. (2001). RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: a negative feedback circuit. Proc. Natl. Acad. Sci. USA *98*, 3056–3061.